

# A new screen for protein interactions reveals that the *Saccharomyces cerevisiae* high mobility group proteins Nhp6A/B are involved in the regulation of the GAL1 promoter

XP-001074698

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Edited by Alexander Varshavsky, California Institute of Technology, Pasadena, CA, and approved October 5, 2000 (received for review August 21, 2000)

The split-ubiquitin assay detects protein interactions *in vivo*. To identify proteins interacting with Gal4p and Tup1p, two transcriptional regulators, we converted the split-ubiquitin assay into a generally applicable screen for binding partners of specific proteins *in vivo*. A library of genomic *Saccharomyces cerevisiae* DNA fragments fused to the N-terminal half of ubiquitin was constructed and transformed into yeast strains carrying either Gal4p or Tup1p as a bait. Both proteins were C-terminally extended by the C-terminal half of ubiquitin followed by a modified Ura3p with an arginine in position 1, a destabilizing residue in the N-end rule pathway. The bait fusion protein alone is stable and enzymatically active. However, upon interaction with its prey, a native-like ubiquitin is reconstituted. R-Ura3p is then cleaved off by the ubiquitin-specific proteases and rapidly degraded by the N-end rule pathway. In both screens, Nhp6B was identified as a protein in close proximity to Gal4p as well as to Tup1p. Direct interaction between either protein and Nhp6B was confirmed by coprecipitation assays. Genetic analysis revealed that Nhp6B, a member of the HMG1 family of DNA-binding proteins, can influence transcriptional activation as well as repression at a specific locus in the chromosome of the yeast *S. cerevisiae*.

The split-ubiquitin method is based on the ability of  $N_{ub}$  and  $C_{ub}$ , the N- and C-terminal halves of ubiquitin, to form a native-like ubiquitin (1). Ubiquitin-specific proteases (UBPs), present in the cytosol and nucleus of all eukaryotic cells, recognize the reconstituted ubiquitin, but not its halves, and cleave off a reporter protein, which had been linked to the C terminus of  $C_{ub}$ . The split-ubiquitin assay (split-Ub) is designed to yield efficient association of  $N_{ub}$  and  $C_{ub}$  only if the two ubiquitin halves are linked to proteins that interact *in vivo*. The assay has been shown to detect interactions between cytosolic proteins, membrane proteins, and transient interactions that occur between transporter and substrate during protein translocation across the membrane of the endoplasmic reticulum *in vivo* (1–4). In addition, split-Ub can also be used to demonstrate interactions between transcription factors (5, 6) because, contrary to the two-hybrid system (7), it is not based on a transcriptional readout.

The *Saccharomyces cerevisiae* GAL1 promoter is a well-studied example of transcriptional regulation by nutrients. When the cells are grown in medium containing galactose as the sole carbon source, GAL1 is activated by Gal4p, which binds specifically to the GAL1 promoter. Gal4p interacts with the holoenzyme component Srb4p, thereby recruiting the transcription apparatus to the GAL1 promoter (8). If the carbon source is switched to glucose, the promoter is repressed by two independently operating mechanisms. Gal80p masks the activation domain of DNA-bound Gal4p, thereby preventing the recruitment of the transcription machinery (9). In addition, the cytosolic repressor Mig1p enters the nucleus (10). Mig1p blocks transcription by recruiting the general corepressor Tup1p to its two sites in the operator region of the GAL1 promoter (11, 12). Because the deletion of SRB10, a member of the RNA-PolII holo-

zyme, reduces transcriptional repression by Tup1p, the repressor is thought to directly influence the transcription machinery (13, 14). However, Tup1p has also been shown to bind to the histones H3 and H4, indicating that the repressor might influence transcription by altering the chromatin structure (15, 16). In addition, there are other chromosomal proteins that are thought to play an architectural role in the formation of the chromatin structure: the proteins of the high mobility group (HMG) (17). Proteins of the HMG1/Y family are necessary for the establishment of the structure of an active promoter: the enhancersome (18). The proteins of the HMG1 family are also involved in the negative regulation of transcription (19–23).

The classical two-hybrid screen (7) is not suitable for the identification of interacting partners of proteins that are involved in either transcriptional activation or repression, nor is this approach suitable for the analysis of protein complexes that cannot be reconstituted in the nucleus. Therefore, we developed a generally applicable technique of screening for binding partners of proteins at any place in the cytosol of the cell. To identify additional proteins involved in the regulation of the GAL1 promoter, we carried out two split-Ub screens with Gal4p and Tup1p as baits.

## Materials and Methods

**Strains and Plasmids.** The *S. cerevisiae* strains used were JD52, JD53, JD55 (24), and NLY2 (25). The NHP6 deletion strains were made by successive deletion of the entire NHP6A and NHP6B ORFs with the help of two knockout constructs based on NKY51 (26). After each knockout, the URA3 gene was recombined out on 5-fluoroorotic acid (FOA) plates, and the *hisG* fragment remained in the place of the NHP6A and NHP6B ORFs. Consistent with previous reports, NHP6 deletion from JD52, JD53, and NLY2 caused temperature sensitivity (27). The NHP6 deletions were complemented by the integrative plasmids ASZ10 (28) and YIplac128 (29) containing PCR fragments of the NHP6A or NHP6B genes, respectively. The TUP1 deletion strains were constructed by first deleting the ADE2 gene of JD52 and JD53. An ADE2-marked PCR fragment containing 60 base pairs of the promoter and terminator sequences of TUP1 was then used to delete the entire TUP1 ORF. The REG1 deletion strains were generated by deleting the entire REG1 ORF with a HIS3-marked knockout vector.

This paper was submitted directly (Track 1) to the PNAS office.

Abbreviations:  $C_{ub}$ , C-terminal half of ubiquitin; FOA, 5-fluoroorotic acid; HMG, high mobility group;  $N_{ub}$ , N-terminal half of ubiquitin; split-Ub, split-ubiquitin assay; UBP, ubiquitin-specific protease; HA, hemagglutinin; GFP, green fluorescent protein; GST, glutathione S-transferase.

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Article published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.250400997. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.250400997.

Genomic DNA was isolated from all *S. cerevisiae* knockout strains, and the deletions of the respective genes were verified by PCR and Southern blotting. The *Escherichia coli* strain used for protein purification was BL21(DE3)LysS (Stratagene). The single-copy  $C_{ub}$ -Rura3p fusion vector has been described previously (2). The  $N_{ub}$  fusion vectors PACNX- $N_{ub}$ IBC and PADNX- $N_{ub}$ IBC are single-copy and multicopy derivatives of PADNS (30). In these vectors, we replaced the ampicillin resistance gene with the chloramphenicol resistance gene and subcloned a PCR fragment encoding the N-terminal half of ubiquitin, a hemagglutinin (HA) tag, and a *Bgl*II site in all three reading frames under the control of the ADH1 promoter. The oligonucleotides used are: GCCAAGCT-TATGCAGATTTTCGTCAAGAC, GCCAGATCTCCAGCGTAATCTGGAACA, GCCAGATCTGCCAGCGTAATCTGGAACA, and GCCAGATCTGCCAGCGTAATCTGGAACA. The single-copy  $C_{ub}$ -RGFP fusion vector was constructed by replacing the *MscI/ApaI* fragment containing the URA3 gene of the  $C_{ub}$ -Rura3p fusion vector with a *StuI/ApaI* PCR fragment containing the DNA encoding the green fluorescent protein (GFP). The oligonucleotides used here are GCCAGGCTCATGAGTAAGGAGAAGAACT and GCCGGGCCCTATTTGTATAGTTCATCCATGC. Following standard procedures, we generated the different fusions by cloning PCR fragments of the respective genes into the  $C_{ub}$  and  $N_{ub}$  fusion vectors. The glutathione S-transferase (GST)-Nhp6B fusion was made by cloning the NHP6B ORF into GEX-5X-1 (Amersham Pharmacia). H<sub>2</sub>HA-Tup1p was constructed by cloning a PCR fragment containing the TUP1 ORF, six histidines, and an HA tag into pET11a (Invitrogen).

**The Split-Ubiquitin Screen.** The  $N_{ub}$  fusion library was made by cloning partially restricted *Sau*3A fragments of the ATCC library 37323 into the *Bgl*II site of PADNX- $N_{ub}$ IBC in all three reading frames. A total of  $3 \times 10^6$  independent colonies were obtained, which suggests that the complexity of the original library ( $8 \times 10^4$ ) was retained. A total of  $5 \times 10^4$  transformants were screened for proteins interacting with Gal4(1-147 + 768-881)- $C_{ub}$ -Rura3p on FOA plates containing 100  $\mu$ M CuSO<sub>4</sub>. Four different clones were isolated, and one of them contained NHP6B. Gal80p was not isolated in this screen. In the screen using Tup1p as the  $C_{ub}$ -Rura3p bait,  $10^5$  transformants were plated on medium containing FOA and 100  $\mu$ M CuSO<sub>4</sub>. Sixteen different clones were isolated, one of them as often as eight times. Two of the other clones isolated were obvious artifacts, encoding Gog5p and the related Ymd8p, small molecule transporters that confer FOA resistance when overexpressed. Yak1p, a kinase involved in cell-cycle regulation, was isolated eight times in the screen with Tup1p. It remains to be tested whether there is a biological significance for the interaction between Tup1p and Yak1p. As for the other clones isolated, their interaction will be tested for biological relevance with the help of mutants. The results will be published separately.

**In Vitro Binding Assays.** The GST-fusion proteins were purified according to the protocol of the manufacturer (Amersham Pharmacia). The H<sub>2</sub>HA-Tup1 protein was loaded onto an Ni column (Amersham Pharmacia) and eluted by increasing concentrations of imidazole. The peak fraction appeared at 250 mM imidazole. *In vitro* binding assays were performed as described (31).

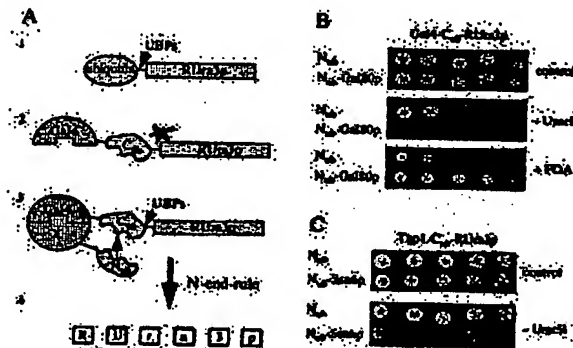
**$\beta$ -Galactosidase Assays.** Yeast strains transformed with the indicated plasmids were grown in liquid culture or on plates and assayed for  $\beta$ -galactosidase activity as described elsewhere (33). The average of at least three independent measurements is shown.

**Western Blots.** Western blot analysis was performed according to ref. 33. Proteins were detected with the anti-HA antibody from Babco (Richmond, CA). The secondary antibody (Bio-Rad) was visualized using the ECL Western blotting detection kit (Amersham Pharmacia) following the manufacturer's protocol.

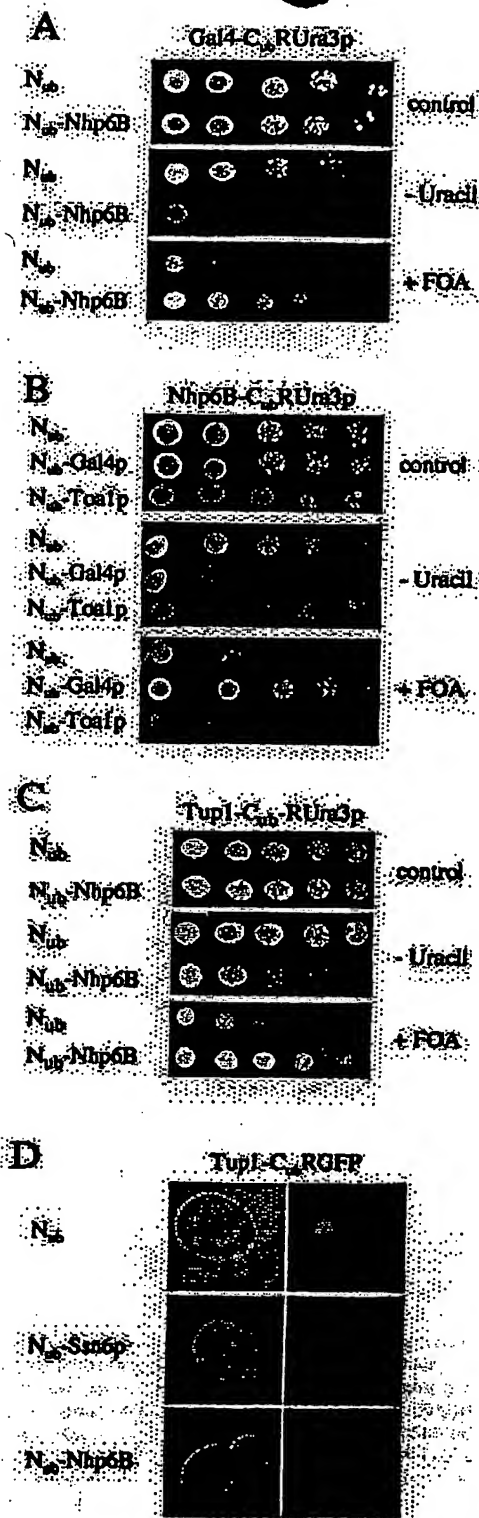
**Northern Blots.** Yeast RNA was isolated as described previously (33) and incubated for 2 min at 60°C in 1× MEN buffer (20 mM Mops/5 mM Na-acetate/1 mM EDTA, pH 7.0) containing 15% (vol/vol) formaldehyde and 50% (vol/vol) formamide. The RNA was loaded on a 0.8% agarose gel [0.8% agarose in 1× MEN buffer + 5% (vol/vol) formaldehyde] and blotted overnight in 0.05 M NaOH onto a nylon membrane (Hybond N<sup>+</sup>, Amersham Pharmacia). The prehybridization was performed for 4 h at 42°C in 0.25 M NaH<sub>2</sub>PO<sub>4</sub>, 0.25 M NaCl, 7% SDS, 1 mM EDTA, 10 mg/liter fish sperm DNA, 5% (wt/vol) PEG 6000, and 25% (vol/vol) formamide. The DNA probe was generated by PCR, purified on an agarose gel, and radioactively labeled by random hexanucleotides (Roche). The hybridization was performed overnight at 42°C, washed in 1× SSC (150 mM NaCl/15 mM Na-citrate) + 0.1% SDS and analyzed by autoradiography.

## Results

**Split-Ub Detects the Interaction Between Gal4p and Gal80p and Between Tup1p and Ssn6p.** To demonstrate that split-Ub can be used to select for protein interactions that occur between transcription factors in *S. cerevisiae*, we first monitored the formation of the well-characterized Gal4p/Gal80p and Ssn6p/Tup1p complexes *in vivo*. Fig. 1A shows the conditional degradation design of the split-Ub system that was used in this study. Ubiquitin fused to a modified Ura3p with an arginine in position 1 (RUra3p) is cleaved by the UBPs (line 1). The free RUra3p is degraded rapidly because arginine is a destabilizing residue in the N-end rule pathway (34) (line 4). A minimal Gal4p, composed of DNA-binding and activation domain only (amino acids 1-147 + 768-881), was fused N-terminally to  $C_{ub}$ , which was C-terminally extended by RUra3p (line 2). The Gal4- $C_{ub}$ -Rura3 fusion protein, which is not recognized by the UBPs, is stable and enzymatically active. *S. cerevisiae* cells transformed with this fusion were therefore uracil prototroph



**Fig. 1.** A system to select for protein interactions *in vivo*. (A) The split-ubiquitin system. Ubiquitin, fused to the N terminus of Ura3p displaying an arginine as its first amino acid (RUra3p), is recognized by the UBPs (line 1). The cleaved RUra3p is rapidly degraded by the N-end rule pathway of protein degradation (line 4). No cleavage of RUra3p takes place if only the C-terminal half of ubiquitin ( $C_{ub}$ ) is fused between Gal4p and RUra3p (line 2). A protein X is attached to the N-terminal half of ubiquitin. If X interacts with Gal4p, the two coupled Ub peptides are forced into close proximity, a ubiquitin-like molecule is reconstituted, and cleavage by the UBPs is observed (line 3). The freed RUra3p reporter is now rapidly degraded by the enzymes of the N-end rule, resulting in uracil auxotrophy and FOA resistance (line 4). (B) Gal4p interacts with Gal80p *in vivo*. Shown are serial dilutions of cells coexpressing  $N_{ub}$  or a  $N_{ub}$ -Gal80p fusion together with Gal4(1-147 + 768-881)- $C_{ub}$ -Rura3p on plates lacking tryptophan and leucine (Top), additionally lacking uracil (Middle), or containing FOA (Bottom). All proteins were expressed from single-copy vectors. (C) Tup1p interacts with Ssn6p *in vivo*. Shown are serial dilutions of cells coexpressing the depicted  $N_{ub}$  and  $C_{ub}$  fusions on plates lacking tryptophan and leucine (Upper) or on plates additionally lacking uracil (Lower). All proteins were expressed from single-copy vectors.



**Fig. 2.** Nhp6B was isolated in two independent split-ubiquitin screens using Gal4p or Tup1p as C<sub>ub</sub>-RUra3p baits. (A) Gal4p interacts with Nhp6B *in vivo*. Serial dilutions of cells coexpressing N<sub>ub</sub> or an N<sub>ub</sub>-Nhp6B fusion together with a fusion of the DNA-binding and activation domains of Gal4(1-147 + 768-881)p to C<sub>ub</sub>-RUra3p were grown on plates lacking tryptophan and leucine (Top), on plates additionally lacking uracil (Middle), or on plates containing FOA (Bottom). N<sub>ub</sub> and N<sub>ub</sub> fused to full-length Nhp6B were expressed from multicopy vectors. (B) The activation domain of Gal4p is sufficient for the interaction with Nhp6B. Serial

and FOA sensitive (Fig. 1B). Gal80p, which is known to bind Gal4p, was fused C-terminally to N<sub>ub</sub> to create N<sub>ub</sub>-Gal80p. The formation of the Gal4p/Gal80p complex is expected to bring N<sub>ub</sub> and C<sub>ub</sub> in close proximity. The two halves of ubiquitin associate into a native-like ubiquitin, and RUra3p is cleaved off by the UBP's (Fig. 1A, line 3). The free RUra3p is degraded rapidly by the enzymes of the N-end rule pathway (Fig. 1A, line 4). Therefore, cells coexpressing N<sub>ub</sub>-Gal80p and Gal4-C<sub>ub</sub>-RUra3p were unable to grow on plates lacking uracil but were able to grow on plates containing FOA (Fig. 1B). The same experiment was repeated with isogenic cells carrying a deletion of the N-end rule pathway recognition component UBR1. These cells are unable to degrade N-end rule substrates like the cleaved RUra3p. As a consequence, the N<sub>ub</sub>-Gal80p/Gal4-C<sub>ub</sub>-RUra3p transformed cells retained their FOA sensitivity and were able to grow on plates lacking uracil (not shown). To test the specificity of the measured interactions, we transformed the Gal4-C<sub>ub</sub>-RUra3p-containing cells with N<sub>ub</sub> alone or N<sub>ub</sub> coupled to the N terminus of either subunits of TFIIA (Fig. 1B and data not shown). In all three cases, no indication for an interaction with Gal4-C<sub>ub</sub>-RUra3p was observed.

Second, a Tup1-C<sub>ub</sub>-RUra3p fusion was constructed. Cells transformed with this fusion were phenotypically uracil prototroph and FOA sensitive (Fig. 1C). Ssn6p, which is known to form a complex with Tup1p, was fused to N<sub>ub</sub> to create N<sub>ub</sub>-Ssn6p. Upon transformation of Tup1-C<sub>ub</sub>-RUra3p containing cells with N<sub>ub</sub>-Ssn6p, the cells became uracil auxotroph and FOA resistant. No indication for an interaction was observed between Tup1-C<sub>ub</sub>-RUra3p and N<sub>ub</sub> or the N<sub>ub</sub> derivatives of either TFIIA subunit (Fig. 1C and data not shown), which demonstrates the specificity of the observed interaction between N<sub>ub</sub>-Ssn6p and Tup1-C<sub>ub</sub>-RUra3p. To verify that the interaction between N<sub>ub</sub>-Ssn6p and Tup1-C<sub>ub</sub>-RUra3p occurred in the nucleus, we replaced the RUra3p reporter in the Tup1p construct with a GFP module that carried the same degradation signal as RUra3p at the N terminus. Inspection of cells coexpressing N<sub>ub</sub> and Tup1-C<sub>ub</sub>-RGFP revealed strong nuclear green fluorescence. When the cells were coexpressing N<sub>ub</sub>-Ssn6p instead of N<sub>ub</sub>, this green fluorescence disappeared (Fig. 2D). This result strongly suggests that the observed interaction between Ssn6p and Tup1p occurs in the nucleus.

**A New Split-Ub-Based Screen Identifies Nhp6 as a Binding Partner of Gal4p and Tup1p.** To reveal new interaction partners of Gal4p or Tup1p, a N<sub>ub</sub> library was constructed by fusing genomic *S. cerevisiae* Sau3A-partially digested DNA fragments in all three reading frames 3' to the N<sub>ub</sub> moiety. The N<sub>ub</sub> library was transformed into a yeast strain that contained Gal4(1-147 + 768-881)-C<sub>ub</sub>-RUra3p and into a yeast strain that contained Tup1-C<sub>ub</sub>-RUra3p as a bait. After selection on FOA, the plasmids were isolated from the colony-forming cells. Only one particular ORF was discovered in both screens (Fig. 2A and C). Because the corresponding gene promised to reveal new insights into the complex regulation of the GAL1 promoter, we focused on this particular clone. The obtained

dilutions of cells coexpressing N<sub>ub</sub>, N<sub>ub</sub> fused to the activation domain of Gal4p (amino acids 768-881; N<sub>ub</sub>-Gal4p), or N<sub>ub</sub> attached to the large subunit of TFIIA (N<sub>ub</sub>-Toa1p) together with Nhp6B-C<sub>ub</sub>-RUra3p were grown on plates lacking tryptophan and leucine (Top), on plates additionally lacking uracil (Middle), or on plates containing FOA (Bottom). N<sub>ub</sub>, N<sub>ub</sub>-Gal4p, and N<sub>ub</sub>-Toa1p were expressed from multicopy vectors. (C) Tup1p interacts with Nhp6B *in vivo*. Serial dilutions of cells coexpressing the depicted N<sub>ub</sub> and C<sub>ub</sub> fusions were grown on plates lacking tryptophan and leucine (Top), on plates additionally lacking uracil (Middle), or on plates containing FOA (Bottom). N<sub>ub</sub> and the clone isolated from the library expressing N<sub>ub</sub>-Nhp6B that lacked the first 22 amino acids of Nhp6B were on multicopy vectors. (D) Tup1-C<sub>ub</sub>-RGFP is located in the nucleus and interacts with N<sub>ub</sub>-Ssn6p and N<sub>ub</sub>-Nhp6B. Cells expressing the depicted fusions from single-copy vectors were analyzed under a Leitz fluorescence microscope with phase contrast (Left) and fluorescence (Right).

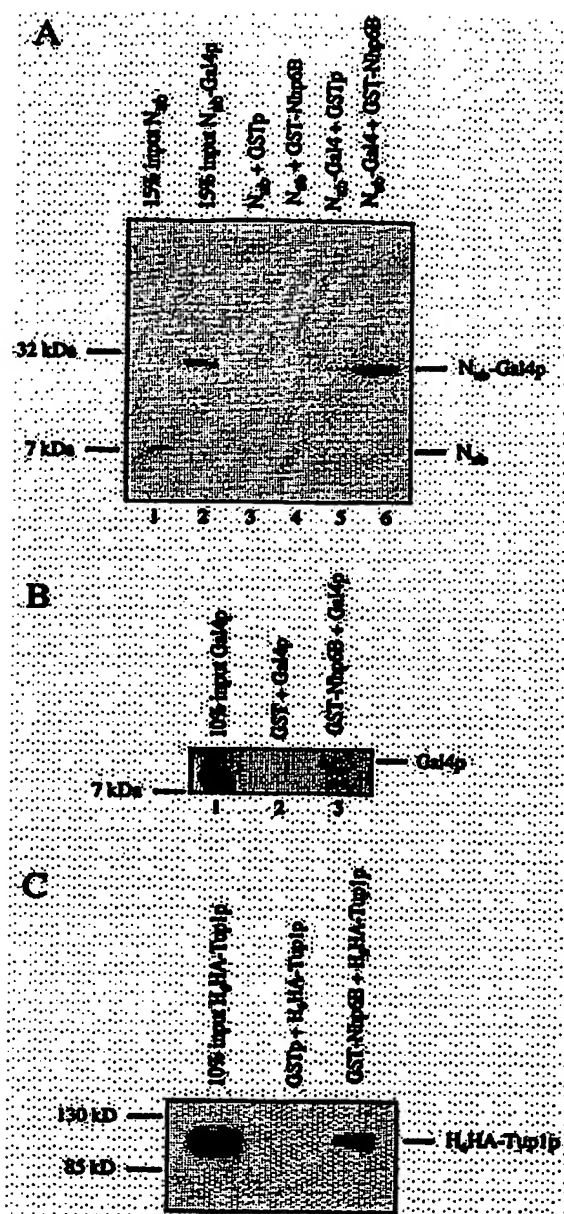
fragment encoded the 77 C-terminal residues of Nhp6B fused in frame to  $N_{ub}$ . Nhp6B is a nonhistone chromosomal protein of the HMG1 family. The isolated fragment lacks the first 22 amino acids of Nhp6B but contains the entire HMG box.

As a control, we tested the interaction between Tup1p and Nhp6B by fluorescence microscopy. Tup1- $C_{ub}$ -RGFP was coexpressed together with  $N_{ub}$  or  $N_{ub}$ -Nhp6B. The bright nuclear fluorescence disappeared upon coexpression with  $N_{ub}$ -Nhp6B. However, the Tup1- $C_{ub}$ -RGFP-induced fluorescence remained in the nucleus upon coexpression with  $N_{ub}$  (Fig. 2D). To find out whether Nhp6B interacts with the DNA-binding or the activation domain of Gal4p, the activation domain of Gal4(768–881) was fused behind  $N_{ub}$ , and the entire reading frame of Nhp6B was cloned in front of  $C_{ub}$ -RURA3p. Compared with the actual screen, the  $N_{ub}$ - $C_{ub}$  arrangement was switched in this experiment. However, the interaction between the two proteins (Fig. 2B) could still be observed. This outcome not only confirmed the result of the screen, it also showed that the DNA-binding domain of Gal4p is not necessary for its interaction with Nhp6B. To test the specificity of the interaction, cells were cotransformed with Nhp6B- $C_{ub}$ -RURA3p and  $N_{ub}$ -Toa1p, the  $N_{ub}$  fusion to the large subunit of TFIIA. Toa1p did not interact with Nhp6B in this assay (Fig. 2B), even though the interaction between the two subunits of TFIIA was readily detected (data not shown).

Split-Ub measures local concentration, but not necessarily a direct interaction between two proteins. To find out whether Gal4p and Nhp6 interact directly, we purified Nhp6B as a GSTp fusion from *E. coli*. We incubated *S. cerevisiae* extracts from cells expressing  $N_{ub}$  or  $N_{ub}$  fused to the activation domain of Gal4p with either GSTp or GST-Nhp6B, and the bound material was precipitated with glutathione beads. Because  $N_{ub}$  and  $N_{ub}$ -Gal4p contained the HA epitope, bound and unbound fractions were probed by anti-HA immunoblotting after SDS/PAGE. The activation domain of Gal4p was specifically precipitated with GST-Nhp6B from the extract (Fig. 3A, lane 6). Also, GST-Nhp6B precipitated the *in vitro* translated activation domain of Gal4p (Fig. 3B, lane 3). To test whether the measured proximity between Tup1p and Nhp6B also reflects a direct protein interaction, we fused six histidines and an HA tag to the N terminus of Tup1p. The obtained  $H_6$ HA-Tup1p was purified from *E. coli* and incubated with purified GSTp or GST-Nhp6B attached to glutathione-Sepharose beads.  $H_6$ HA-Tup1p was only detected after SDS/PAGE by the anti HA antibody in the bound fraction of the GST-Nhp6B beads and not in the bound fraction of the GSTp beads (Fig. 3C).

Nhp6A is almost identical to Nhp6B. The presence of either protein is sufficient for proper cell growth, which indicates that Nhp6B can functionally replace Nhp6A. In contrast to Nhp6B, expression of Nhp6A from the ADH1 promoter on a multicopy vector is toxic for the cells. This explains why Nhp6A could not be isolated from the  $N_{ub}$  library. However, when we expressed the  $N_{ub}$ -Nhp6 fusions from single-copy vectors, we found that Nhp6A interacts with Gal4- $C_{ub}$ -RURA3p and Tup1- $C_{ub}$ -RURA3p as efficiently as  $N_{ub}$ -Nhp6B (data not shown). The functional redundancy of the two Nhp6 proteins seems to be reflected by the redundancy of their interactions. The interactions were observed independently of Gal80p and with and without  $CuSO_4$  in the medium (data not shown).

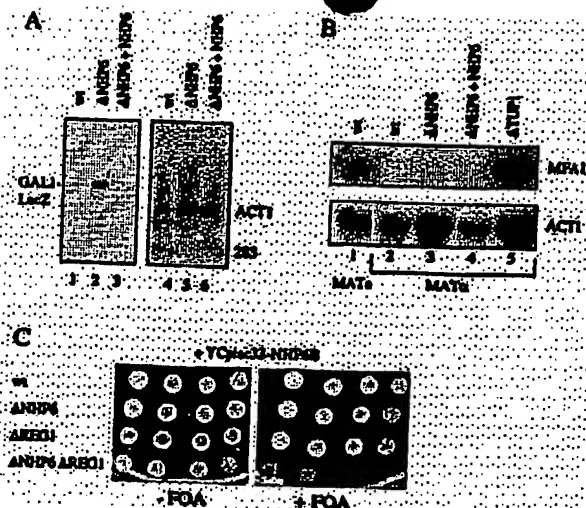
**The Interaction of Nhp6 with Tup1p Influences the Repression of the GAL1 Promoter.** To learn more about the physiological relevance of the interaction between Nhp6 and Gal4p and between Nhp6 and Tup1p, we deleted the complete reading frames of both NHP6 genes in several strains. Because Tup1p is known to repress the GAL1 promoter in glucose-containing medium (35), we tested the effect of the NHP6 double deletion on the transcription of a GAL1-LacZ reporter gene. When the cells were grown in glucose, we measured 0.51  $\beta$ -galactosidase units for the wild-type strain and 5.3 units for the NHP6 deletion



**Fig. 3.** Nhp6B interacts with Gal4p and Tup1p *in vitro*. (A) Gal4p coprecipitates together with Nhp6B from *S. cerevisiae* extracts. Extracts from *S. cerevisiae* cells expressing  $N_{ub}$  or  $N_{ub}$ -Gal4p (amino acids 768–881) from multicopy vectors were incubated with GSTp or GST-Nhp6B purified from *E. coli* on glutathione beads. Coprecipitated proteins were separated on an SDS gel and visualized on a Western blot with an anti-HA antibody with the help of an HA tag present in the  $N_{ub}$  moiety. (B) *In vitro* translated Gal4p interacts with Nhp6B. The activation domain of Gal4p (amino acids 768–881) was radiolabeled by *in vitro* translation and incubated with a bacterially purified GSTp or a GST-Nhp6B fusion bound to glutathione beads. Coprecipitated proteins were visualized by autoradiography. A truncated form of the activation domain of Gal4p, migrating faster in the SDS gel, showed no interaction with GST-Nhp6B. (C) Purified Tup1p interacts with purified Nhp6B. A  $H_6$ HA-Tup1p fusion was purified on an Ni column and incubated with purified GSTp or GST-Nhp6B on glutathione beads. Coprecipitated  $H_6$ HA-Tup1p was visualized on a Western blot with an anti-HA antibody.

strain. The isogenic strain deleted for TUP1 yielded 12.7 units. We performed a Northern blot with a LacZ probe and demonstrated that the loss of glucose repression took place at the level



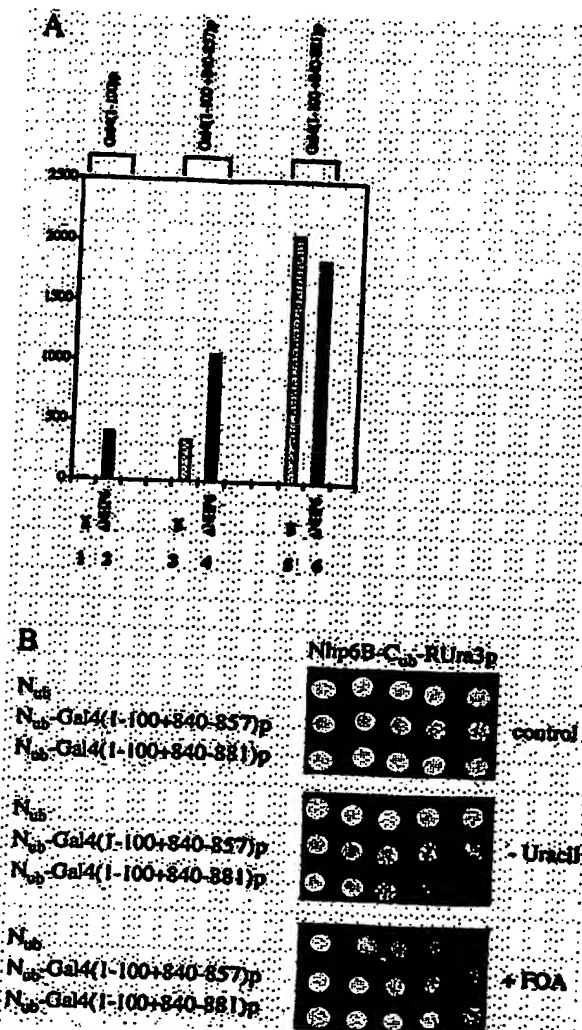


**Fig. 4.** The interaction between Nhp6B and Tup1p is biologically relevant. (A) Nhp6 is necessary for glucose repression of the GAL1 promoter. RNA was prepared from the depicted strains carrying a GAL1-LacZ fusion integrated at the GAL1 locus. JD53 was used as wild-type parental strain (lanes 1 and 4). The  $\Delta$ NHP6 strain was derived from JD53 that lacks NHP6A and NHP6B (lanes 2 and 5). In the strain  $\Delta$ NHP6 + NHP6 (lanes 3 and 6), NHP6A and NHP6B had been reintegrated into the original loci. Equal amounts of total RNA were loaded as confirmed by ethidium bromide staining (not shown) and background hybridization to the 28 S rRNA (Right). The Northern blot was probed with a LacZ probe (lanes 1–3) and with an ACT1 probe (lanes 4–6). We consistently saw a slight increase in the level of ACT1 mRNA in the  $\Delta$ NHP6 strain. (B) Nhp6 is not necessary for  $\alpha$ 2p repression. RNA was prepared from the depicted strains, and the Northern blot was probed with an MFA1 probe (Upper) or with an ACT1 probe (Lower). In lane 1, RNA was isolated from JD52, a MAT $\alpha$  strain. In lane 2, RNA was isolated from JD53, which was used as wild-type parental MAT $\alpha$  strain. Lane 3 contained RNA from JD53 lacking NHP6A and NHP6B ( $\Delta$ NHP6). For lane 4, NHP6A and NHP6B had been reintegrated into the original loci ( $\Delta$ NHP6 + NHP6). Lane 5 contained RNA from JD53 lacking TUP1 ( $\Delta$ TUP1). (C) NHP6 and REG1 deletions are synthetically lethal. Shown are serial dilutions of the depicted *S. cerevisiae* strains carrying a URA3-marked Nhp6B expression plasmid (YCPac33-NHP6B) on medium lacking or containing FOA.

of transcription (Fig. 4A). The increased amount of the GAL1-LacZ mRNA in the NHP6 deletion strain (compare lanes 1 and 2) were reduced to wild-type levels upon reintegration of NHP6 (lane 3). We also tested the expression of the glucose-repressed SUC2 promoter in our deletion strains. As has been shown for the GAL1-LacZ transcription, the integrated SUC2-LacZ reporter showed reduced reduction of glucose repression in the NHP6 deletion strain as well as in the strain lacking TUP1 (data not shown). Besides regulating glucose-responsive genes, Tup1p is also involved in the repression of MFA1 in MAT $\alpha$  cells. Interestingly, Nhp6 does not seem to be involved in the Tup1-mediated  $\alpha$ 2p repression (Fig. 4B). Although the deletion of TUP1 resulted in derepression of MFA1 in MAT $\alpha$  cells, the deletion of NHP6 had no effect (compare lanes 2, 3, and 5). A similar pattern was observed for the expression of the  $\alpha$ 2-regulated STE2. A STE2-LacZ fusion was up-regulated in the TUP1 deletion strain (data not shown). Cells that are deficient for Tup1 display a flocculent phenotype (36). This phenotype was not observed for cells lacking Nhp6. These observations indicate that Nhp6 acts together with Tup1 specifically on the glucose-regulated promoters GAL1 and SUC2. However, unlike Tup1p, Nhp6 is not involved in the repression of the mating type-specific promoters MFA1 and STE2.

**Synthetic Lethality Between NHP6 and REG1.** Not to rely exclusively on experiments with artificial promoter fusion constructs, we

tried to delete REG1. REG1 causes the degradation of glucose-repressed mRNAs by XRN1 in glucose (37). A REG1 deletion should therefore allow to measure the effect of the NHP6 deletion on the transcription of the natural GAL1 and SUC2 genes. However, several independent strains chromosomally deficient for NHP6A, NHP6B, and REG1, which carried NHP6B on a URA3-marked plasmid, were unable to lose this plasmid and therefore unable to grow on FOA (Fig. 4C). This experiment shows that simultaneous deletion of REG1 and NHP6 is lethal to the cells and provides an independent link between NHP6 and glucose repression.



**Fig. 5.** A truncated form of Gal4p, which displays an impaired interaction with Nhp6B, results in elevated levels of transcription upon deletion of NHP6. (A) Deleting NHP6 results in increased levels of transcription of a GAL1-LacZ reporter by a truncated form of Gal4p. Strains of the indicated genotype carrying a GAL1-LacZ reporter were transformed with the depicted expression plasmids. Arbitrary units of  $\beta$ -galactosidase activity are shown for the parental NLY2 strain, which lacks GAL4 and GAL80 in lanes 1, 3, and 5. The  $\beta$ -galactosidase activities of NLY2 cells additionally lacking NHP6A and NHP6B are shown in lanes 2, 4, and 6. Cells were grown in liquid glucose medium, and  $\beta$ -galactosidase activity was determined as described (33). Numbers were measured in triplicate, and standard deviations were less than 20%. All Gal4p derivatives were expressed from single-copy vectors. (B) Truncating the minimal activation domain of Gal4p results in decreased interaction with Nhp6B. Serial dilutions of cells coexpressing the depicted N<sub>ub</sub> and C<sub>ub</sub> fusions were grown on plates lacking tryptophan and leucine (Top), on plates additionally lacking uracil (Middle), or on plates containing FOA (Bottom). All proteins were expressed from single-copy vectors.

The interaction of Nhp6 with Gal4p influences the activation of the GAL1 Promoter. In contrast to published findings, we could not measure a decrease in the activation potential of Gal4p in cells lacking NHP6 (27). We reasoned that Gal4p, as an activator of transcription, might be simply too strong to yield a significant effect of Nhp6 on the transcription of the reporter genes. We therefore compared the ability of Gal4p derivatives that lacked parts of the activation domain to stimulate transcription in strains containing or lacking NHP6. The Gal4p derivatives were expressed as  $N_{ub}$  fusions from the constitutive ADH1 promoter. This enabled us to test the same molecule for both transcriptional activation and interaction *in vivo*. NHP6 was deleted from the *S. cerevisiae* strain NLY2, which is deficient for GAL4 and GAL80 (25). A GAL1-LacZ fusion was integrated into the GAL1 locus of the NLY2 wild-type and NHP6 deletion strains. The strains were transformed with the plasmids expressing the Gal4p derivatives, and cells were grown in glucose. Fig. 5A shows transcriptional activation of a GAL1-LacZ fusion by three different  $N_{ub}$ -Gal4p derivatives. Increasing the size of the deletion within the activation domain corresponded to a decrease in the transcription of the LacZ reporter, and this effect was seen independently of NHP6. However, there was a clear difference in the extent of activation between the NHP6-containing and NHP6-lacking strains. The  $N_{ub}$ -Gal4p derivative that has no or only a severely truncated activation domain stimulated transcription from the GAL1 promoter significantly better in a strain that lacks NHP6 (compare lanes 3 and 4). This difference was not observed for the  $N_{ub}$ -Gal4p fusion that harbored the complete activation domain (compare lanes 5 and 6). The ability to activate transcription in the strain carrying NHP6 correlated with the ability of the two  $N_{ub}$ -Gal4p derivatives to interact with Nhp6B- $C_{ub}$ -Rura3p. The Gal4p derivative with the truncated activation domain interacted less efficiently with Nhp6B than the protein with the intact activation domain (Fig. 5B). We suggest that one additional function of the activation domain of Gal4p is to contact and to remove Nhp6 or remodel its position on the chromatin structure.

## Discussion

Yeast two-hybrid screens have been successfully used to isolate binding partners of proteins fused to a DNA-binding domain (7). However, proteins that activate or repress transcription in *S. cerevisiae* cannot be used as baits because the signal of the two-hybrid screen itself is based on the transcriptional readout of a reporter protein. The split-ubiquitin system makes use of the facilitated reassociation of the two ubiquitin halves and the subse-

quent cleavage by the UBPs. As a consequence, transcriptional regulators do not interfere with the readout and can be used as baits in a screen. This rationale was confirmed in the work presented here. In a two-step approach, we first showed that split-Ub can monitor the interaction between transcription factors by following the formation of the Gal4p/Gal80p and of the Ssn6p/Tup1p complexes *in vivo*. Cells expressing a Gal4- $C_{ub}$ -Rura3p fusion or a Tup1- $C_{ub}$ -Rura3p fusion display a *ura*<sup>-</sup> phenotype only if an  $N_{ub}$ -Gal80p or an  $N_{ub}$ -Ssn6p fusion is coexpressed. Second, we have shown that split-Ub can be used to screen  $N_{ub}$  fusion libraries for proteins that interact with a given  $C_{ub}$ -Rura3p bait. Using the two known regulators of the GAL1 promoter, Gal4p and Tup1p, as  $C_{ub}$ -Rura3p baits, we have isolated the HMG box of the chromosomal protein Nhp6B in both screens. Interaction was also observed for full-length Nhp6B, which demonstrates that at least in this case, structural constraints are not limiting the split-Ub system. Because split-Ub measures the local concentration of the  $N_{ub}$ - and  $C_{ub}$ -coupled proteins, it was important to biochemically determine the nature of this proximity. Using GST pull-down assays, a direct interaction between Nhp6 and Tup1p and between Nhp6 and Gal4p was established. Furthermore, we have shown that the observed protein interactions are biologically relevant for the regulation of the GAL1 promoter.

The approach introduced here will also allow to screen for binding partners of proteins that are not localized in the nucleus. There are now different  $C_{ub}$ -Rura3 fusion proteins available that are cytosolic or directed to the membrane of the endoplasmic reticulum, the outer mitochondrial membrane, the membrane of the peroxisome, or the plasma membrane (2) (J. H. Eckert and N.J., unpublished data). The scarcity of methods to analyze membrane proteins makes this system particularly attractive.

To be able to confirm the localization of the  $C_{ub}$ -modified proteins, we have created an N-end rule-sensitive GFP reporter for the split-Ub system. Using this assay, Tup1- $C_{ub}$ -RGFP localized in the nucleus of the cells. The fluorescence disappears upon introduction of the  $N_{ub}$  versions of the two Tup1p binding partners Ssn6p and Nhp6B. This feature of the new reporter will give us the opportunity to better follow the dynamics of protein interactions in living cells or monitor signals that induce or terminate a specific protein interaction.

We thank Alexander Szyroki for help with the GFP pictures and Benno Müller-Hill for critical comments on the manuscript. This work was supported by grants from the Bundesministerium für Forschung und Bildung and the Max-Planck-Gesellschaft (to N.J. and N.L.).

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